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(OPA-2) Containing Stealth Liposomes

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<b>13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information)</b> The investigational effort was focused on improving the antagonism of anticholinesterase organophosphorus agents by alteration of the physiological disposition of the antagonists. Various approaches were employed, but the primary emphasis was placed on prolonging its pharmacological activity by enhancing its duration of action. This was accomplished by forming a cyclodextrin adducts of various pyridinium derivatives. An alternative method was to encapsulate the enzymes organophosphorus acid anhydrolase, OPAA, and organophosphorus hydrolase, OPH, into liposomes and lastly to attach the enzymes to nanotechnology derived particles we called nanostructures and we have termed this area as nanobiotechnology. These approaches have been able to protect and treat animals exposed to the organism phosphoric agents by up to 1000 lethal doses (LD-50). This is the highest protection against an organophosphorus agent ever reported. By using new stable nanostructures and recombinant enzymes which are both relatively easy to produce in mass production procedures by molding molecular biology with nanotechnology. Plans were being projected to examine sarin and saman by these nanobiotechnology approaches.				
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## **'PYRIDINIUM' APPENDED CYCLODEXTRIN DERIVATIVES**

## 'Pyridinium' Appended Cyclodextrin Derivatives

### A. Introduction

#### A. Introduction

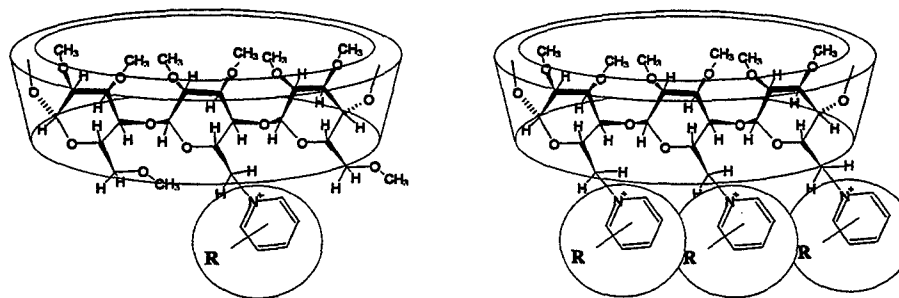
##### 1. Scope

Direct reactions of mono- and polyfunctionalized  $\beta$ -cyclodextrins having free hydroxyl groups on the secondary hydroxyl rim of cyclodextrins have been failed to produce the desired derivatives. The strong basicity of both PAMs and pyridine aldehydes favors the formation of 3,6-anhydro cyclodextrins.

The reactions of free aldehyde with an activated cyclodextrin results in very complex reaction mixture which could not be separated even with column chromatography in inert atmosphere. In order to avoid the side reactions of pyridine aldehyde derivative ethyl acetals were used as temporary protecting group. The regeneration of the aldehyde function could be carried out under very mild conditions and the formation of the monoxime was performed under conventional reaction conditions.

Reactions of the acetal protected pyridinium aldehydes have not resulted in the desired products. The 3,6-anhydroglucopyranose unit formation was again the favored reaction.

In order to avoid the free secondary hydroxyls, a new strategy was applied. It is known that methylated cyclodextrins are well water soluble despite the hydrophobic methyl substitution of all hydroxyls. Two types of activated methylated cyclodextrin derivatives have been prepared: Permethylated 6-monodeoxy-6-monohalogeno-beta-cyclodextrin (*Type A*) and 2,3-permethylated 6-perdeoxy-6-perhalogeno-beta-cyclodextrin (*Type B*).



*Type A*

and

*Type B* conjugates

In case of advantageous physicochemical and biological properties of the *Type B* conjugates of the targeted antidotes, the corresponding alpha- and gamma-cyclodextrin conjugates can be prepared, too. *Type A* conjugates for alpha- and gamma-cyclodextrins can be prepared only on hundred milligram scale.

Preparation of *Type A* cyclodextrin derivatives was performed by the following way: 6-monotosylated cyclodextrin was prepared; the excellent leaving group was exchanged to azido group resulting in a chemically stable 6-monodeoxy-6-monoazido- $\beta$ -cyclodextrin. Methylation of the azido derivative in dimethyl sulfoxide/sodium hydroxide/methyl iodide system resulted in 6-monodeoxy-6-monoazido-heptakis(2,3-di-O-methyl)-hexakis(6-O-methyl)- $\beta$ -cyclodextrin. Photolysis and borohydride reduction regenerated the single hydroxyl group on the primary side, which could be easily converted to the corresponding 6-monodeoxy-6-monobromo-permethyated  $\beta$ -cyclodextrin. Reaction with the temporary protected aldehyde resulted in the desired intermediary product. Deprotection of the aldehyde and the formation of the corresponding aldoxime could be performed by the usual way.

Preparation of *Type B* cyclodextrin derivatives was performed by the following way: Temporarily protecting of the primary hydroxyls of cyclodextrins was done by *tert*butyldimethylsilylation (TBDMSi). TBDMSiCl was reacted with of all commercially available cyclodextrins in very dry pyridine and the products were purified by flash chromatography. Methylation of the TBDMSi derivative in dimethyl sulfoxide/sodium hydroxide/methyl iodide system resulted in hexakis-, octakis-, and heptakis(2,3-di-O-methyl-6-O-TBDMSi)- $\alpha$ -,  $\gamma$ -, and  $\beta$ -cyclodextrin. Removal of TBDMSi groups was achieved by hydrolysis under mild acidic conditions. Halogenations of 2,3-permethyated cyclodextrins were carried out by usual triphenylphosphine induced halogenation. The halogenated intermediary product was reacted with the protected aldehyde resulting in a complex mixture. Chromatographic purification of the *Type B* protected aldehyde, deprotection of the aldehyde and their oximation resulted in the targeted molecules.

## 2. Purpose

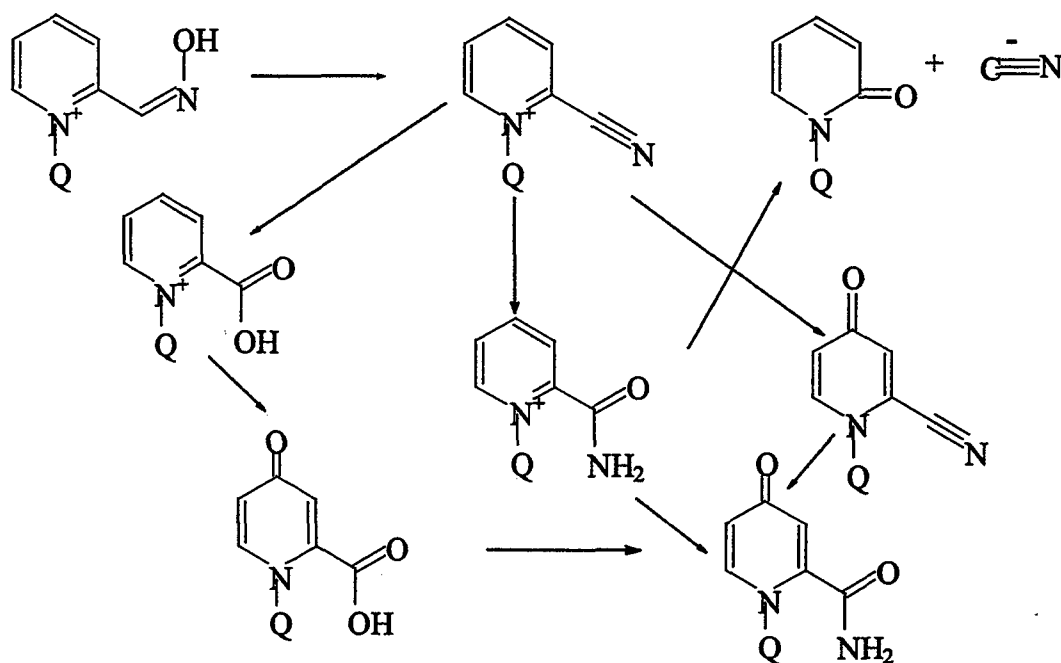
1. To find appropriate cyclodextrin derivatives for the preparation of pyridinium aldehyde oxime conjugates.
2. To improve the preparation methods of activated cyclodextrins
3. To prepare an appropriate pyridinium-cyclodextrin conjugate.
4. To prepare the pyridinium aldehyde oxime conjugates.

## B. Body

About 70 papers have been published on the preparation of various pyridinium moiety containing cyclodextrin derivatives for studying their redox properties. Less than 10 of these papers are dealing with cyclodextrin-pyridinium derivatives, the others are describing 2-5 carbon spacer between the CD and the pyridinium group that are attached to the alkyl chain (viologens). The described derivatives are almost exclusively  $\beta$ -cyclodextrin derivatives. The substituents are on the primary hydroxyl side, due to the stereochemical course of the substitution reactions (on the secondary side the well-defined substitution is more difficult and may lead to configuration change at C(2)/C(3)). The reason is simple: monofunctionalization of  $\alpha$ - and  $\gamma$ -cyclodextrins are more difficult (being less rigid structures than the  $\beta$ -cyclodextrin).

The currently used pyridinium aldoxime type antidotes are very well water soluble which decreases its bioavailability due to their short residence times, see *Scheme 1*.

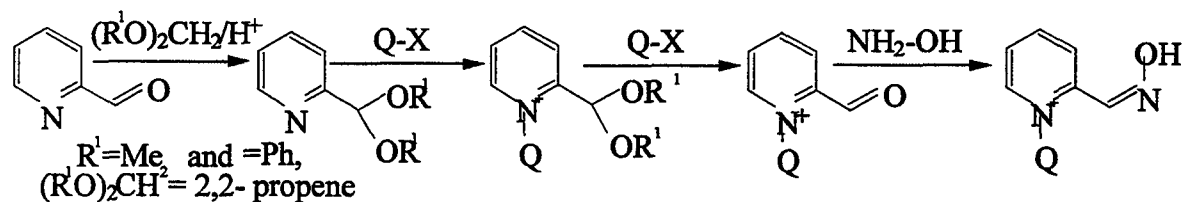
It is expected that the cyclodextrin/pyridinium aldoximes will have enhanced residence time in a living organism.



**Scheme 1.** Decomposition of 2-PAM quaternary salts

*Expected difficulties of direct reaction of a pyridinium compound and activated cyclodextrins*

1. Under basic conditions the formation of 3,6-anhydro- $\beta$ -cyclodextrins is preferred if free hydroxyls exist on the secondary side.
2. Nitrogen of an aldoxime may be more basic than an aromatic nitrogen.
3. As a consequence of the more expressed basicity of aldoxime moiety, the temporary protection of the aldehyde part is expected, as it can be seen on *Scheme 2*.

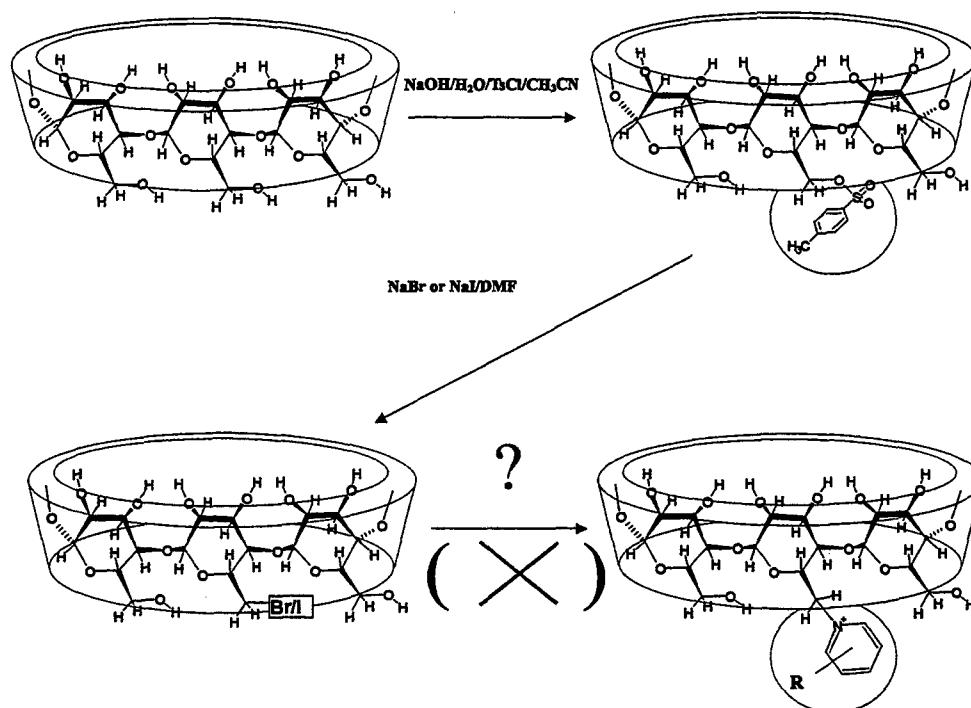


**Scheme 2.** Alternative synthesis of CD-2-PAM

#### A. Preparation of *Type A* conjugates

Usually, the preparation of derivatives are following the  $\beta$ -cyclodextrin-6-tosylate  $\rightarrow$  [6-deoxy-6-iodo- $\beta$ -cyclodextrin]  $\rightarrow$  6-deoxy-6-pyridinium- $\beta$ -cyclodextrin pathway, as can be seen on *Scheme 3*.



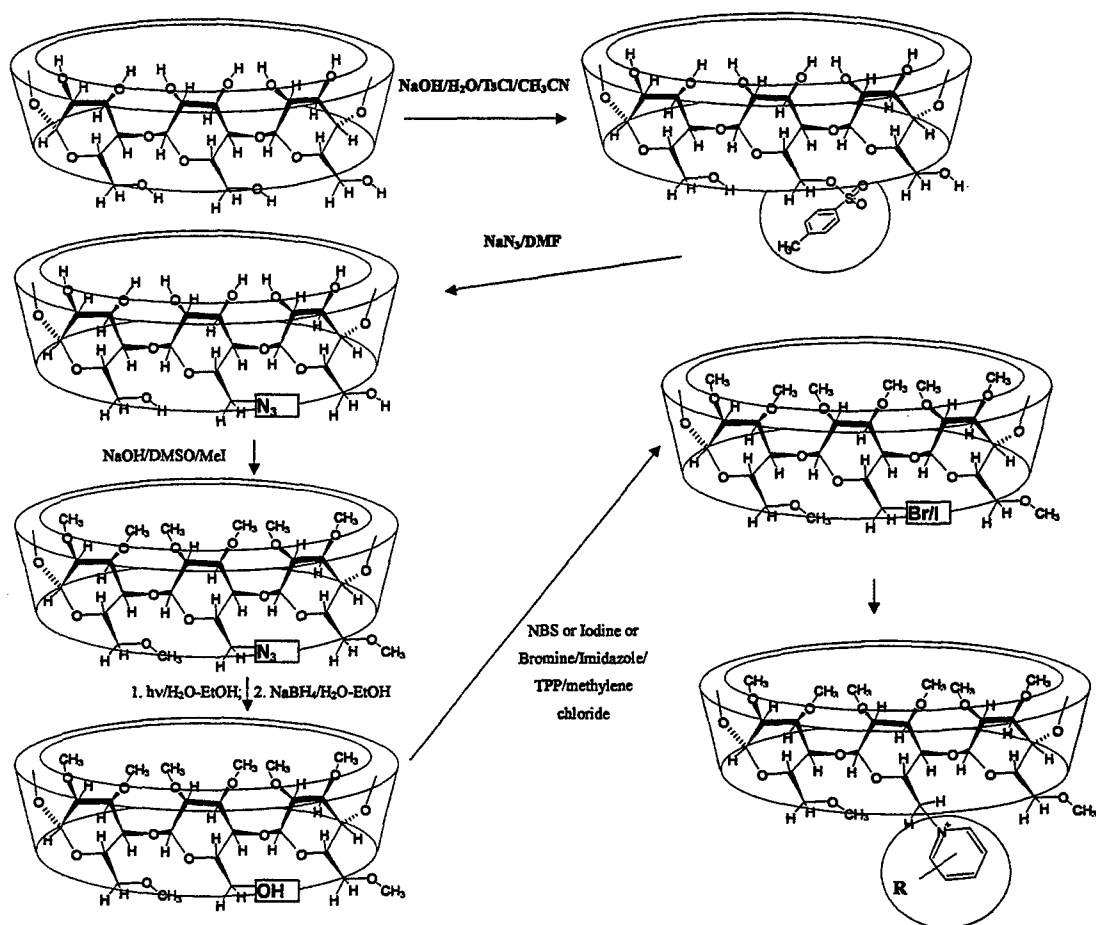


*Scheme 3.: Planned synthesis of Cyclodextrin-PAM conjugates*

#### *Known problems with the current methods*

1. Selective derivatization of  $\beta$ -cyclodextrin can be performed only on the primary hydroxyl side with a more or less acceptable yield.
2. The yield of the preparation of 6-monotosyl- $\beta$ -cyclodextrin is low (not more than 40-45%). The use of pyridine as solvent results in hardly removable impurities by the multiple substitution.
3. The exchange of the tosyl group is necessary, because the sluggish exchange rate of the tosyl group results in the formation of 3,6-monoanhydro- $\beta$ -cyclodextrin. Iodine or bromine is a suitable group to increase the yield of the pyridinium compound.
4. Chromatography is necessary in the preparation that may result in a considerable loss of the prepared material.

An alternative way to eliminate the abovementioned difficulties is the exchange of hydroxyl groups to methoxy groups, as it is demonstrated on *Scheme 4*.



**Scheme 4.:** Preparation of *Type A* cyclodextrin-PAM conjugates

In case of *Type A* conjugates the major difficulties can be avoided by using a permethylated monoactivated cyclodextrin. In order to achieve an absolutely selective monoactivation, the tosyl->azide exchange is necessary. The prepared azide is stable enough in the methylation reaction but relatively easy to regenerate the hydroxyls via photolysis. The prepared monohydroxy cyclodextrin can be activated (bromo or iodo substitution) using triphenylphosphine/imidazole and bromine or iodine. Due to the lack of free secondary hydroxyls the formed activated cyclodextrin can be used as a conventional alkyl halogenide in order to prepare the corresponding pyridinium compounds.

#### B. Preparation of *Type B* conjugates

The following pathway is suitable for the preparation of all the commercially available ( $\alpha$ -,  $\beta$ -, and  $\gamma$ -) cyclodextrins.

6-Perdeoxy-6-perhalogeno-cyclodextrins  $\rightarrow$  6-deoxy-6-pyridinium- $\beta$ -cyclodextrin pathway was selected for the first attempts. If the negative expectations with the reaction become reality, the following alternative synthetic way is planned:

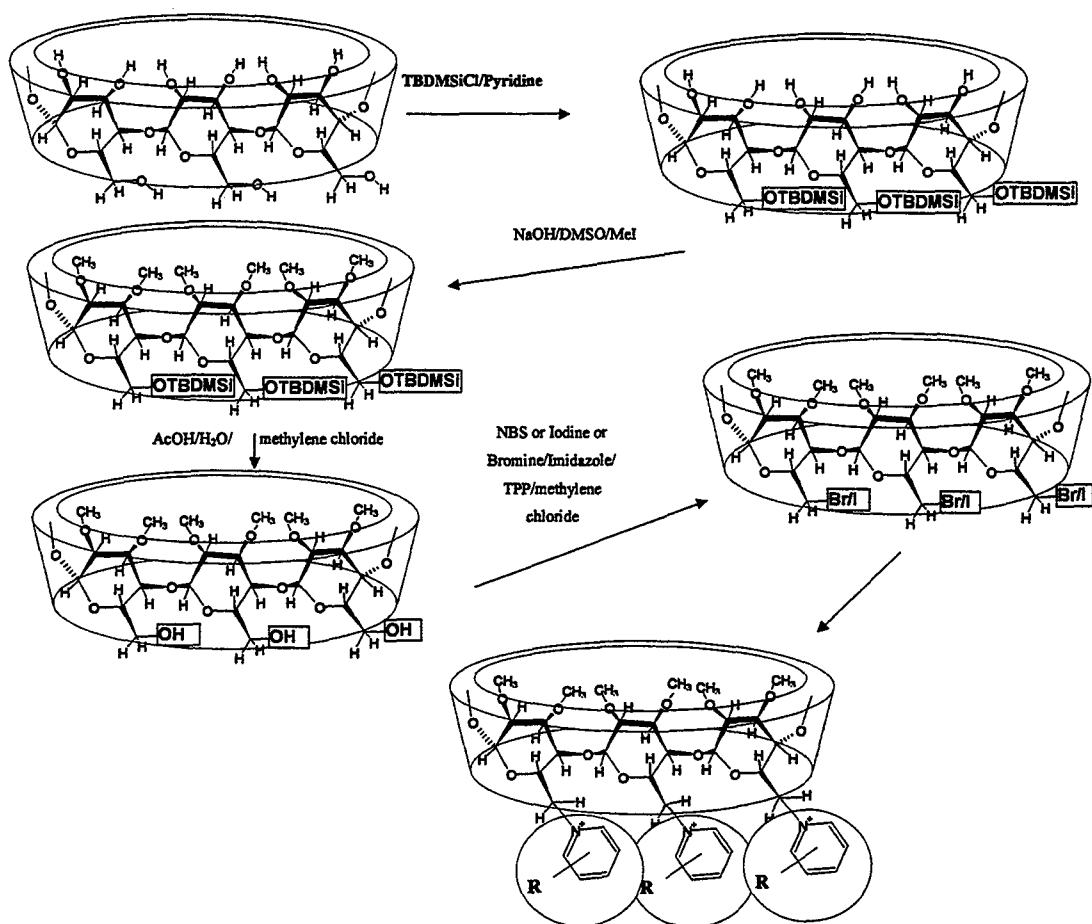
Per-6-TBDMSi- $\beta$ -cyclodextrin  $\rightarrow$  Per-6-TBDMSi-2,3-methyl- $\beta$ -cyclodextrin  $\rightarrow$  Per-2,3-methyl- $\beta$ -cyclodextrin  $\rightarrow$  Per-6-bromo/iodo-2,3-methyl- $\beta$ -cyclodextrin  $\rightarrow$  Per-6-pyridinium-2,3-methyl- $\beta$ -cyclodextrin.

Selective hydroxyl- $\rightarrow$ halogen exchange of the cyclodextrins' primary hydroxyls is well known. The resulted 6-perhalogeno cyclodextrins are practically insoluble in the most organic solvents what makes the purification relatively easy. Unfortunately, the formation of anhydroglucopyranose units may be not surprising in this case as well, the same difficulties are also expected as in case of *Type A* derivatives.

In order to avoid the formation of anhydroglucopyranose units, the exchange of hydroxyls to methoxy groups is advantageous. Unfortunately, the 6-perazido cyclodextrins have poor solubilities in common organic solvents and methylation of all the secondary hydroxyls does not result in dramatic changes in solubilities in alcohol/water mixtures which are the preferred reaction media for photolysis.

Selective protection of primary hydroxyls of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -cyclodextrins are well known, but chromatography is necessary during the purification that make synthesis time consuming. An additional disadvantage of this protecting group is its large molecular weight and possible primary  $\rightarrow$  secondary hydroxyl migration. Deprotection of the methylated TBDMSi-cyclodextrin derivatives are carried out under mild acidic conditions and due to the nature of this protecting group, resulted in considerable loss of mass (about 60-75 %).

The prepared 2,3-di-O-methyl-6-perhydroxy cyclodextrins can be activated (bromo- or iodo substitution) using triphenylphosphine/imidazole and bromine or iodine. Due to the lack of free secondary hydroxyls the formed activated cyclodextrin can be used as a conventional alkyl halogenide in order to prepare the corresponding pyridinium compounds. An additional advantage of this type of conjugates is the larger number of pyridinium moieties on a cyclodextrin which can be enhance the efficiency of the conjugate as it can be seen on *Scheme 5*.



*Scheme 5.: Preparation of Type B cyclodextrin-PAM conjugates*

## Results:

The following pyridine aldoximes are involved in our study:

2-pyridinealdehyde monoxime (2-PAM)

4-pyridinealdehyde monoxime (4-PAM)

4,4'-bipyridyl-2,2'-dialdehyde dioxime (bipyridyl dialdoxime)

Toxogonine

HI6

TMB4

### *Preparation of Type A pyridinealdehyde oximes*

Preparation method of 6-O-monotosyl- $\beta$ -cyclodextrin was improved. Using toluene as complexant resulted in higher yield (~50 % isolated yield instead of 5-25 %). Yields of tosyl->bromo/iodo exchanges were around 90 %, so the overall yield is around 40 % which is acceptable on larger production scale, too.

Direct reactions of 6-monodeoxy-6-moniodo- and 6-monobromo-, and 6-O-monotosyl- $\beta$ -cyclodextrins with 2-PAM and 4-PAM resulted in no pyridinium quaternary salts but the mono-3,6-anhydro- $\beta$ -cyclodextrin.

In order to make only one substitutable nitrogen and reduce the basicity 2- and 4-pyridinealdehyde diethyl acetals were prepared. Reactions of these acetals resulted in very complex reaction mixtures which could not be separated. In these cases the formation of 3,6-monoanhydro- $\beta$ -cyclodextrin was preferred, as well. Preparation of the pyridinealdehyde acetals could be performed with ~50 % yield, which is acceptable at this stage of work.

Heptakis(6-deoxy-6-bromo/iodo)- $\beta$ -cyclodextrins were prepared with ~80 % isolated yields. Unfortunately, both the PAM and pyridinium acetals favored the various 3,6-anhydro- $\beta$ -cyclodextrin formation.

In order to avoid the anhydroglucopyranose unit formations the methylation of futile hydroxyls was targeted. In case of monosubstitution, the tosyl group was changed to azido group, which is very stable under usual alkylation conditions. Permethylation of 6-monoazido-6-monodeoxy- $\beta$ -cyclodextrin was prepared with 75 % isolated yield by the reaction of dried monoazido-6-monodeoxy- $\beta$ -cyclodextrin and methyl iodide in dimethylsulfoxide with finely powdered sodium hydroxide. Overall yield from  $\beta$ -cyclodextrin is ~33 %.

Regeneration of the hydroxyl group was performed by photolysis. It was found that in aqueous alcoholic solutions the photolytic transformation of C-N<sub>3</sub> bond to aldehyde is faster. The limit of water concentration depends on solubility of the azido compound. Immediate reduction of the formed aldehyde with sodium borohydride resulted in hexakis(2,3,6-tri-O-methyl)-2,3-di-O-methyl- $\beta$ -cyclodextrin in 80 % isolated yield. Overall yield from  $\beta$ -cyclodextrin is ~25 %.

The monohydroxy permethylated cyclodextrin is soluble in most of organic solvents and the classical halogenation of a primary hydroxyl with triphenylphosphine/imidazole/ $\text{BR}_2$  or  $\text{I}_2$  or NBS can be applied, with ~85 % isolated yield. Overall yield from  $\beta$ -cyclodextrin is ~20 %.

Reaction of the activated cyclodextrin with the various pyridinealdehyde diethyl acetals to obtain *Type A* derivatives could be performed with moderate yields, 20-40 % after chromatography in dimethyl formamide solution at elevated temperatures (75-125 °C). Overall yield of *Type A* pyridinealdehyde diethyl acetals from  $\beta$ -cyclodextrin is ~5-10 %.

Hydrolysis of the *Type A* pyridinium aldehydes were carried out in aqueous alcoholic solutions by acetic acid, with practically quantitative yields. Overall yield from  $\beta$ -cyclodextrin is ~5-10 %. It was found that the prepared quaternary pyridinium aldehydes are unstable, therefore the immediate oxime preparation is necessary.

*Type A* oximes were obtained by the reaction of the freshly prepared pyridinium aldehyde and hydroxylamine in basic aqueous alcoholic solutions with ~90 % isolated yield. Overall yield from  $\beta$ -cyclodextrin is ~5 %.

#### *Preparation of Type B pyridinealdehyde oximes*

Preparation of heptakis(6-hydroxy-2,3-di-O-methyl)- $\beta$ -cyclodextrin *via* similar reaction path seemed to be unreasonable due to the bad aqueous solubility of perazido cyclodextrins, so for the protection primary hydroxyls a different reaction scheme (*Scheme 5.*) was chosen.

Preparation of heptakis(6-O-TBDMSi)- $\beta$ -cyclodextrin was carried out in very dry pyridine and the flash chromatographed crude product resulted in 50 % isolated yield of the targeted cyclodextrin derivative.

Methylation of the TBDMSi- $\beta$ -cyclodextrin derivative was carried out under the same conditions as used for the permethylated 6-monoazido derivative resulted in the heptakis(2,3-di-O-methyl-6-O-TBDMSi)- $\beta$ -cyclodextrin in ~60 % isolated yield. Overall yield from  $\beta$ -cyclodextrin is ~30 %.

Desilylation was carried out in tetrahydrofurane/acetic acid/water mixtures with ~90 % isolated yield. Overall yield from  $\beta$ -cyclodextrin is ~30 %.

Halogenations of heptakis(2,3-di-O-methyl)- $\beta$ -cyclodextrin were carried out under the same conditions as used for the permethylated 6-monohydroxy derivative, and resulted in the heptakis(2,3-di-O-methyl-6-deoxy-6-bromo/iododo)- $\beta$ -cyclodextrin in ~60 % isolated yield. Overall yield from  $\beta$ -cyclodextrin is ~20 %.

Reaction of the activated cyclodextrin with the various pyridinealdehyde diethyl acetals to obtain *Type B* derivatives could be performed with moderate yields, 5-20 % after chromatography in dimethyl formamide solution at elevated temperatures (75-125 °C). Overall yield of *Type B* pyridinealdehyde diethyl acetals from  $\beta$ -cyclodextrin is ~2-5 %.

Hydrolysis of the *Type A* pyridinium aldehydes were carried out in aqueous alcoholic solutions by acetic acid, with practically quantitative yields. Overall yield from  $\beta$ -cyclodextrin is ~2-5 %. It was found that the prepared quaternary pyridinium aldehydes are unstable, therefore the immediate oxime preparation is necessary.

*Type B* oximes were obtained by the reaction of the freshly prepared pyridinium aldehyde and hydroxylamine in basic aqueous alcoholic solutions with ~90 % isolated yield. Overall yield from  $\beta$ -cyclodextrin is ~1-5 %.

Type B derivatives are containing cyclodextrin derivatives that are not fully substituted with the appropriate pyridinealdehyde oximes.

#### C. Key Research Accomplished

A novel enzyme-nanodelivery system has been developed to antagonize the toxic organophosphorus (OP) molecule in the biologic system. This complex stabilizes and delivers the enzyme for a long period of time and also enhances the specific activity of the enzyme. This represents one of the first attachments of a recombinant enzyme into a nanocapsule to form an active therapeutic drug, which is superior to present organophosphorus antidotes. In this case, a poly (2-ethyloxazoline) dendritic polymer based nanocapsule is utilized for protein delivery. This polymer was synthesized through a one pot polymerization process with a diameter of 60 nanometers. Biocompatibility with this new class of dendritic polymers represents one of the most active biomedical applications termed biomedical nanoscience. Our model proteins are recombinant organophosphorus hydrolyzing enzymes, organophosphorus acid anhydrolase (OPAA) organophosphorus hydrolase (OPH). The enzymes hydrolyze the substrate,

diisopropylfluorophosphate (DFP) and paraoxon, respectively. The classic antidotal combination, pralidoxime (2-PAM) and atropine, only blocks the pharmacological effects and reactivates the OP inhibited enzyme. Only through the use of this dendritic enzyme polymer complex is the OP agent absorbed into the body actually degraded. The nanoencapsulation technology could serve as a platform technology to deliver and to activate other protein based therapeutic agents.

#### D. Reportable Outcomes

This is a 10 month report. The funds to test these compounds were delayed by interagency transfer of funds within the U.S. Army laboratories to test these compounds. These funds were returned at the end of contract.

#### E. Conclusion

The OP antidote, 2-PAM, was a dramatic biochemical accomplishment. However, the physiological disposition of the agonist versus the antagonist was not a major consideration in the molecular design. The 2-PAM is short action and sarin is relatively long acting. Reintoxication should not occur if the physiological disposition of 2-PAM was taken into consideration.



## II. Title - Nanobiotechnology of OP antagonism with nanostructural encapsulation of OPAA and OPH

### A. Introduction

#### 1. Purpose

Nanotechnology and recombinant enzyme is a new conceptual approach to develop antidotes against environmental toxins. The objective is to explore the biological potential of nanotechnology by using dendrimers and nanocapsule as these nanostructures can serve as delivery system for drugs and proteins. Water soluble drug, protein carrier, and more importantly they appear to be well tolerated with the relatively minimal toxicity in an *in vivo* system. These studies have broad relevance to environmental toxicology, agricultures, and occupational medicine.

#### 2. Scope

- 1) To entrap organophosphorous (OP) hydrolyzing enzymes into nanostructure and to study the effect on preventing or reversing the treatment of OP poisoning.
- 2) To explore a series of nanostructures in addition to the hyperbranched 2-ethyloxazoline based polymeric nanocapsule.
- 3) To studies OP's affects with *in vivo* models such as mice and zebrafish and models *in vitro* such as PC12 cells and retinal ganglion cells to gain more insights into the mechanism and site(s) of OP action.

#### 3. Hypothesis

It is proposed that the use of paraxonase and organophosphorous acid anhydrolase (OPAA) in new nanotechnology. Nanocapsule will enhance the degradation of OP agent and the body of conceptual importance is the observation that some of these nanocapsules are acting not merely as a drug carrier but they actually enhance the specific activity of the enzyme by a mechanism not clearly understood.

#### 4. Background and Significance

The OP compounds are the leading cause of agriculture hazards from pesticides. The OP agents account for 35% of the mortality and morbidity from all pesticides. EPA announced the manufactures of diazinon and other pesticides will be terminated in the US by 2003. These are hazardous not only occupational workers, but the general population: particular children. For example, parathion was used as an aerial spray over Seattle in the 1980's and Malathion is still being used as an aerial spray over Southern California to treat fruit fly problems. OP reagents are also common household and industrial pesticide that causes toxic and teratogenic effects at low concentrations. In human, chronic exposures of OP such as Malathion causes many neuro-ophthalmolgic impairments including optic neuropathy, degeneration of retina, defective vertical smooth pursuit and myopia.

These manifestations were most profound among children. There is increasing concern that exposure of OP may produce aberrant

behaviors involving young children because a common household OP pesticide (chlorpyrifos) alters neonatal brain development and elicits behavioral abnormalities that emerge after a period of apparent normality in human and animal models. In rat studies, a single subcutaneous injection of 100 µg/kg fenthion decreased carbachol-stimulated release of inositol phosphates in retina and inhibited cholinesterase activity in both frontal cortex and retina.

These effects in retina persisted for two months. These biochemical changes observed following a single exposure of OP were specific to retina. The effects of OP on the embryonic nervous system of a model teleost medaka, *Oryzias latipes* had retinal cell necrosis and also acetylcholinesterase activity reduction within whole embryos and in homogenates of retina. Histology of embryos after micromolar exposure of diazinon showed a small focus of necrotic cells within the inner nuclear layer and individually pyknotic cells were found in the retinal ganglion layer.

Zebrafish is an attractive model to study OP effects in fish because they are easily manipulated genetically; many physiological tools and behavioral assays are established. Furthermore, zebrafish are very sensitive to visual cues that all blinded fish do not survive to adulthood. Without visual cues, most fish can not feed and as a result die. The retina is the first visual processing center where it extracts and encodes specific features of the visual scene such as local contrast, color information and movement. As a sensory tissue, the retina displays an extraordinary ability to adapt to ambient light conditions as well as it is most accessible part of the brain. Thus all known neuronal pathways such as excitatory (glutamatergic), inhibitory (GABAergic) and synaptic (glycinergic) pathways are better understood in retina than in the cortex. In the retina, acetylcholinesterase is found in the ganglion cell layer and both in inner and outer nuclear layers (Miyata et al 1974). Muscarinic cholinergic receptors reside in the inner nuclear layer and the ganglion cell layer.

The retinal muscarinic receptors are thought to be located on a subset of both amacrine and retinal ganglion cells and the cholinergic amacrine cells are thought to modulate the ganglion cell response to changes in illumination thus enhancing the detection of motion by the retina.

Any change in the functional response of these cells may lead to alteration in the resolution of spatial motion and/or the ganglion cell response to change in illumination.

Long term changes occurred in electroretinogram of the rat retina following acute exposure to (OP) fenthion (Imai, 1975), while chronic exposure produced permanent retinal degeneration in laboratory animals and humans.

These independent models indicate a strong association between OP exposure and retina toxicity.

Development of specific antidotes against poisoning in general has been disappointing. For example, there are only six specific antidotes which can protect against toxicants by over five LD<sub>50</sub> (excluding antitoxins). The incidents of OP poisoning are not officially decreasing despite changes to less toxic congeners. If a new OP antidote can be developed to degrade the OP agents, this may greatly decrease the incidence of OP poisoning. Pralidoxine (2-PAM) and atropine, and official US antidotal combination do not remove the OP from the body. If such an agent can be developed to degrade OP in vivo, and then the antidotal potential can be greatly improved. Preliminary studies on nanocapsule with recombinant paraoxonase show that paraoxon protection can be enhanced over 1000 LD<sub>50</sub>.

Recently new nanotechnological polymeric dendrimers and nanocapsules have been synthesized. The nanocapsules are larger nanostructures with a larger internal void space and we are using to encapsulate paraoxonase. With the rapid progress of molecular biology, more drugs will be developed as large molecules especially polypeptides and proteins. The physiologic disposition of these proteins in vivo is unfavorable. However by placing these active compounds within a protective environment, such as nanocapsules, this will extend the therapeutic efficacy of proteins by prolonging their effect from hours to days. The use of fast catalytic recombinant enzymes with nanocapsules represents a new approach and I can protect over 1000 LD<sub>50</sub> doses of paraoxon. Although 2-PAM and atropine are the US antidotes of OP intoxication, the recombinant enzyme in a nanobiotechnology carrier system appear to be vastly superior.

Various starburst polyamidoamine CPAMAM polymers and other dendrimers have been encapsulated with peptides, oligonucleotides and porphyrins. Most of these gene transfer and nucleotide studies were done merely at the tissue culture level. To my present knowledge, this probably represents the first report of nanotechnology, producing an efficacious antidotal compound in vivo (mice).

The development of nanotechnology materials is rapidly emerging. There is no precise definition for nanostructures, but it would generally involve molecular structures that range from 5 to 100 nm. These would include covalently linked polymers containing various molecular architectures as well as products formed through a self-assembly template process. However, almost all of the existing synthetic methodologies involve multistep synthesis. My research will focus initially on poly (2-ethylloxazoline) based nanocapsules as they can be produced through a one-pot polymerization process using commercially available monomers. They also possess unique properties that may be potentially useful for a wide variety of biomedical application involving protein and drug-carrying nanostructures. This research represents only a small exploratory application of nanostructures in vitro and in vivo. Employment of water soluble high

molecular weight nanocapsule approach with recombinant paraoxonase and OPAA shows promise against OP intoxication and preliminary results suggest that these nanocapsules to be a promising approach.

#### B. Body

The use of nanostructures with paraoxonase and OPAA to antagonize OP was underway before I became acquainted with this group at the liposome research laboratory at UCSF. My initial input was the use of in vitro system such as the PC12 cell culture system and employ apoptosis assays in these studies. At UCSF, the use of nanostructures was a late finding of this group. My colleagues encouraged me to continue with this research. They would provide me with a supply of OPAA, Ops and the nanostructures to continue these studies. I shall continue to complete in vivo studies and also conduct the in vitro studies using the PC12 culture system.

This offer by my colleagues in this group provided me an opportunity to apply all my trainings to a protected toxicological and environmental problem. My training in neurobiology, physiology and signal transduction blend in with the OP intoxication, as the predominant toxicity is in the CNS and one of the target tissues is the retina. This permits me to conduct low level chronic toxicity in the retina and reversal of this toxicity.

#### C. Research Accomplished

For brevity in discussion, the pharmacological, pathological and toxicological properties of the nanocapsule alone will be addressed. These preliminary studies were presented at the American Chemical Society Symposium on Nanotechnology in San Diego in 2001. It was pointed out at that time that this may represent the first truly efficacious drug therapy derived from nanotechnology.

Recent studies of OPs have identified several targets in addition to acetylcholinesterase. In fact it is unclear which targets are important for the toxic effects of OPs. For example trimethylpropane phosphate reduces of chloride current induced by GABA; thus, it inhibits a spontaneous GABAergic transmission and also decreases in spontaneous inhibitory postsynaptic currents (ipsp). Another OP, chlorpyrifos oxon, potentiates diacylglycerol (DAG) signaling in CHOK1 cells by inhibiting DAG lipase and by activating ERK 44/42 (extracellular signal regulated kinases); OPs are known to block DAG lipases and monoacylglycerol lipases which indirectly increase the amount of DAG present in these cells.

In rat frontal cortex, OPs (Paraoxon, Malaoxon and chlorpyrifos oxon in nM to  $\mu$ M concentrations) inhibit forskolin-stimulated cAMP formation and this effect was blocked by the muscarinic antagonist atropine which suggests the involvement of second messenger systems coupled to Muscarinic 2/4 receptors,

In the rat hippocampus (Mundy et al 1993), in a human parotid salivary cell-line HSY and SH-SY5Y human neuroblastoma cells, a small dose of 0.1 or 1 nM OP (Paraoxon) affects the phosphoinositide signaling pathway coupled with muscarinic receptors. In spite of many years of research, the actual molecular, enzymatic or other targets for their neurotoxin action are not yet clearly understood. In order to develop a specific antidote for organophosphate, it is important to identify all signal transduction pathways and proteins that are directly affected.

#### D. Reportable Outcomes

This report covers the last twelve months. These results are still preliminary, but have been favorably received. Recently, this research was selected to be presented at

- 1) International Symposium on Chemical Warfare in Stockholm, Sweden.
- 2) National Symposium of the American Chemical Society on Nanotechnology.
- 3) National Symposium of Enzymes sponsored by the Department of Defense.

Preliminary collaboration to examine these antidotes with sarin and soman was delayed, as the protocol to accomplish inter-agency intramural transfer of funds was delayed beyond the time frame of our contract. Funds allocated for these studies were returned to DOD.

#### E. Conclusions

The use of nanobiotechnology has arrived with regard to treatment and prophylaxis against some chemical warfare agents (cyanide and OP agents). Concerning the use of OPAA and OPH in nanostructure...it is far superior to any reports in scientific literature and with no reportable side effects. It has far superior efficacy and without the severe physical disability of the present antidotes. Moreover, both the nanostructure and the enzymes OPAA and OPH can be mass produced. These are the only antidotal agents which reported an efficacy of protection 1000 lethal doses. Also, the duration of action of this antidote is for days and weeks rather than for a few hours.

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